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# Effects of Common Estuarine Pollutants on the Immune Reactions of Tunicates

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**Abstract.** Tunicates are filter-feeding estuarine and marine animals that are frequently exposed to chronic environmental pollution. This study demonstrates that exposure to low-level (*i.e.*, below the threshold of acute lethality) contamination with tributyltin, creosote, and copper can have substantial effects on natural immune reactions in tunicates. Sublethal doses of toxicants administered either *in vitro* or *in vivo* profoundly affected phagocytosis, cellular cytotoxicity, and hematopoietic cell proliferation. Effects were not always inhibitory, and responses often varied depending on the route of toxicant administration. The data suggest that pollutants can activate cascades of cellular processes and compensatory mechanisms, as well as directly inhibiting some of the responses tested. Some evidence indicates that toxicants exert their effects by altering the relative frequencies of circulatory hemocytes.

## Introduction

Marine invertebrates can be profoundly affected by aquatic pollution. Detrimental effects have been identified using tests for acute lethality (*e.g.*, 96-h $LD_{50}$), toxicant bioaccumulation, anatomical and biochemical aberration, or altered biodiversity and abundance (Giam and Ray, 1987; Landis and Yu, 1995; Peakall, 1992). However, there is relatively little information regarding the effects of environmental contamination on natural immune reactions in invertebrates, even though modulation of the immune system may dramatically alter populations by affecting their resistance to infection (Anderson *et al.*, 1990; McCarthy and Shugart, 1990; Roales and Perlmutter, 1977; Sarot and Perlmutter, 1976; Stebbing, 1985). Some evidence suggests that the effects of decreased disease resistance resulting from low-level, chronic pollution may not be reflected accurately in assays that test acute lethality. For instance, heavy metal or polychlorinated biphenyl contamination in worms can significantly inhibit lysozyme activity, wound healing, phagocytosis, rosette formation, and tissue transplantation rejection at concentrations that are not acutely lethal (Cooper and Roch, 1992; Fitzpatrick *et al.*, 1992; Rodrigues-Grau *et al.*, 1989).

The aim of the current study is to demonstrate that subacute contamination with three common estuarine pollutants—the antifouling agents tributyltin (TBT), copper, and creosote—can significantly affect the immunological defenses of tunicates. Tunicates (Urochordata, Ascidiacea) are aquatic filter-feeding invertebrates that are ubiquitous components of estuarine and coastal marine systems (Berrill, 1950; Goodbody, 1974). They occur in large populations on marinas, moorings, and wharves that are often subjected to chronic pollution, particularly from antifouling treatments. The effects of pollution are likely to be compounded in tunicates by their filter-feeding lifestyle. Large volumes of potentially polluted water pass over the sensitive endothelial surfaces of the pharynx (up to 100 l of water per day), greatly increasing the propensity of tunicates to absorb toxicants (Goodbody, 1974).

Here, we test the effects of toxicants on a number of well-characterized assays of immunological reactivity in tunicates. Those assays quantify hemopoietic cell proliferation (Raftos and Cooper, 1991; Raftos *et al.*, 1991), phagocytosis (Beck *et al.*, 1993; Kelly *et al.*, 1993), and cellular cytotoxicity (Parrinello *et al.*, 1993; Peddie and Smith, 1993, 1994). Tunicates lack adaptive antipatho-

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*Abbreviations:* FSW, filtered seawater; MAC, marine anticoagulant; MS, marine saline; PAH, polycyclic aromatic hydrocarbon; RRBC, rabbit red blood cells; TBT, tributyltin; TBS, Tris-buffered saline.

genic defense mechanisms that are analogous to the mammalian adaptive immune system (Raftos, 1994). Hence, innate (or natural) immunological reactions, such as phagocytosis and cellular cytotoxicity, are probably the major protective responses of these animals.

## Materials and Methods

### *Tunicates*

Specimens of *Styela plicata* were collected from two sites on Sydney Harbor, Australia (Balmoral Beach and Birkenhead Point). After collection, tunicates were maintained in 40-l aquaria filled with seawater. Aquaria were held at 14°C under constant aeration. These conditions can maintain *S. plicata* for up to 2 months with limited mortality. *In vivo* exposures were conducted in partitioned aquaria containing 40 l of seawater per compartment.

### *Toxicants*

Tributyltin oxide and copper sulfate were purchased from ICN Chemicals (Sydney, NSW, Australia) and creosote was obtained from BBC Pty Ltd (Chatswood, NSW, Australia). Tributyltin and copper were diluted directly in filtered seawater (FSW; 0.45-$\mu$m filtration). Creosote was prepared as a saturated stock solution in FSW by vigorously agitating 1% v/v creosote in toxicant-free, filtered seawater overnight and then filtering the solution (0.45-$\mu$m) to remove insoluble material. Concentrations of creosote are cited as percentages (v/v) of filtered, saturated creosote solutions. Chemical analysis (Australian Analytical Laboratories, Asquith, NSW, Australia) revealed that 5% v/v of a saturated solution of creosote contained 1 mg/l total polycyclic aromatic hydrocarbons (PAH) (napthalene, 280 $\mu$g/l; anthracene, 250 $\mu$g/l; phenanthrene, 150 $\mu$g/l; acenapthene, 93 $\mu$g/l; remaining PAHs $< 80$ $\mu$g/l). No PAHs (practical quantitation limit = 1 $\mu$g/l) were detected in the normal seawater used in aquaria.

### *Treatment protocols*

Two forms of toxicant treatment were applied. Tunicate cells were exposed to toxicants *in vitro* to assess effects on isolated tissues, and live tunicates were exposed to toxicants in aquaria to identify effects that were derived from interactions between organ or physiological systems. Where possible, the effects of toxicants were tested over a range of doses and at a number of time points for exposure periods of up to 9 days. For brevity, only representative data reflecting trends in both dose response and kinetic analysis are presented here.

### *Dosages*

The ranges of doses used for the three toxicants were selected to incorporate concentrations that yielded significant differences from nontreated controls in at least one of the assays tested. Copper was the only compound that proved to be acutely toxic at high doses. All tunicates ($n = 8$) died within 8 days of exposure to $\geq 5$ $\mu$g/ml copper. Mortality was assessed by the sensitivity of tunicates to touch (failure to retract siphons) and by an analysis of hemocyte (blood cell) viability. None of the other doses tested, including $<5$ $\mu$g/ml copper, caused mortality within 20 days ($n = 4$ per dose).

### *Hemocyte harvesting and manipulation*

Hemocytes were harvested from incisions in the buccal siphons of *S. plicata*. The exuded hemolymph (blood) was collected in equal volumes of ice-cold marine anticoagulant buffer (MAC; 0.1 *M* glucose, 15 m*M* trisodium citrate, 13 m*M* citric acid, 10 m*M* EDTA, 0.45 *M* NaCl, pH 7.0; Peddie and Smith, 1994) or FSW. Debris and cell aggregates were removed from the hemolymph by sedimentation for 5 min ($1 \times g$). As required, hemocytes were washed by centrifugation ($400 \times g$, 5 min, 4°C) through either MAC or FSW.

### *Cell viability and morphology*

Hemocyte viabilities and the relative frequencies of distinct hemocyte subpopulations were determined using a FACScan flow cytometer with an argon-ion laser tuned to 488 nm (Becton Dickenson, Mountain View, CA). Hemocytes for flow cytometry were obtained either by bleeding tunicates that had been exposed to toxicants in aquaria or by harvesting cells that had migrated from cultured pharyngeal explants during *in vitro* exposures ("emigrant hemocytes"; see *Proliferative activity of toxicant-treated tunicate cells* section). In viability studies, hemocytes ($1 \times 10^6$/ml) were stained with ethidium bromide (0.1% v/v) immediately prior to analysis. Dead cells were detected by their increased red (800 nm) fluorescence reflecting the intercalation of ethidium bromide into cellular DNA. The relative frequencies of distinct hemocyte subpopulations were determined by analyzing forward angle versus 90° light-scatter plots.

### *Proliferative activity of toxicant-treated tunicate cells*

To quantify hemopoietic cell proliferation, tunicate tissue cultures were established by excising small portions ($2 \times 2$ mm) of the pharynx for explant culture in tunicate tissue culture medium (T-RPMI; Raftos and Cooper, 1990). Each 100 ml of T-RPMI contained 10 ml RPMI-1640 tissue culture medium (with sodium bicar-

bonate, without L-glutamine; Sigma Chemicals, St. Louis, MO), 1 ml 20% w/v NaCl, 1 ml antibiotic stock solution (4 mg/ml streptomycin sulfate, $10^3$ IU/ml penicillin sulfate), 100 $\mu$l 1 *M* L-glutamine, and 88 ml FSW. Cultures were maintained at 15°C without $CO_2$ supplementation. Under these conditions explant cultures normally maintain cell viability and function for up to 70 days (Raftos and Cooper, 1990).

For *in vitro* exposure trials, explants were cultured for up to 8 days in 96-well flat-bottomed tissue culture plates containing 200 $\mu$l/well T-RPMI and various concentrations of toxicants. Explants were moved to fresh medium every 2–4 days. After appropriate exposure periods of up to 8 days, explants were incubated (overnight, 15°C) with 18.5 MBq/ml $^3$H-thymidine (Amersham, NSW, Australia, 740 GBq/mmol). Non-incorporated $^3$H-thymidine was removed after incubation by extensive washing in FSW. Explants were then digested (37°C, overnight) in 2.0% w/v trypsin (Sigma Chemicals) to facilitate liquid scintillation counting in Ecolite scintillation cocktail (ICN, Seven Hills, NSW, Australia) (Raftos *et al.*, 1991).

Explants from tunicates exposed to toxicants in aquaria were excised, incubated immediately in T-RPMI containing 18.5 MBq/ml $^3$H-thymidine (overnight, 15°C), and then washed and digested as described above.

### *Phagocytic activity of toxicant-exposed hemocytes*

To test the phagocytic activity of tunicates that had been exposed to toxicants in aquaria, hemocytes from treated tunicates were harvested into FSW and their densities adjusted to $3 \times 10^6$ cells/ml without washing. For *in vitro* exposures, toxicants were added to hemocyte suspensions harvested from nonexposed tunicates ($3 \times 10^6$ cells/ml in T-RPMI). Aliquots (200 $\mu$l) of hemocyte suspensions were cultured (15°C) on autoclaved glass coverslips (22 × 22 mm) for either 2 h (aquarium exposures) or overnight (*in vitro* exposures). Adherent hemocytes were washed with 400 $\mu$l FSW before being overlaid with 50 $\mu$l yeast (Baker's yeast type II, Sigma Chemicals; $5 \times 10^6$ yeast/ml) that had been prepared and opsonized with *S. plicata* plasma according to the method of Beck *et al.* (1993). Noningested yeast were removed by extensive washing with FSW after a 30-min incubation (15°C), and phagocytic activity was quantified microscopically (Beck *et al.*, 1993; Kelly *et al.*, 1993).

### *Cytotoxic activity of treated cells*

Hemocytes from tunicates that had been exposed to toxicants in aquaria were harvested in MAC and tested immediately in cytotoxicity assays. For *in vitro* exposures, hemocytes were harvested from nontreated tunicates and cultured overnight (15°C) in T-RPMI containing various concentrations of toxicants before being tested for cytotoxic activity.

Cytotoxic activities of hemocytes were tested in two assays that used either K562 human chronic myelogenous leukemia cells or rabbit red blood cells (RRBC) as targets. The capacity of hemocytes to kill K-562 cells was assessed by a modification of the method of Peddie and Smith (1993). Hemocyte suspensions were adjusted to $4 \times 10^7$ cells/ml in MAC; 50–100 $\mu$l of these hemocyte suspensions were then mixed in round-bottomed 5-ml flow cytometry tubes with equal volumes of K-562 cells ($4 \times 10^6$ cells/ml) suspended in marine saline (MS; 12 m*M* $CaCl_2 \cdot 6H_2O$, 11 m*M* KCl, 26 m*M* $MgCl_2 \cdot 6H_2O$, 45 m*M* Tris, 38 m*M* HCl, 400 m*M* NaCl, pH 7.4). The cell mixtures were incubated at 15°C for 90 min, stained with ethidium bromide and then immediately tested, using a FACScan flow cytometer, for the uptake of red fluorescence (800 nm). Specific cytotoxic activities were calculated as the percentage of dead K562 cells in a particular sample minus the percentage of dead cells in controls that contained K562 cells but no hemocytes. K562 cells were obtained from the American Type Tissue Culture Collection (Rockville, MD) and were grown in RPMI-1640 tissue culture medium. Immediately prior to their use in cytotoxicity assays, K562 cells were conditioned to the high tonicity of MS by incubation (30 min, 20°C) in an intermediate saline solution (12 m*M* $CaCl_2 \cdot 6H_2O$, 11 m*M* KCl, 26 m*M* $MgCl_2 \cdot 6H_2O$, 45 m*M* Tris, 38 m*M* HCl, 300 m*M* NaCl, pH 7.4; Peddie and Smith, 1993).

The ability of hemocytes to lyse RRBC was quantified by the method of Parrinello *et al.* (1993). Hemocytes were washed once through Tris-buffered saline (TBS; 10 m*M* Tris, 150 m*M* NaCl, pH 7.4) and resuspended in TBS supplemented with 10 m*M* $CaCl_2$ (TBS-Ca) to yield $2 \times 10^6$ cells/ml. One-hundred microliters per well of the suspensions were then incubated (60 min, 37°C) with an equal volume of RRBC ($4 \times 10^7$ cells/ml in TBS-Ca) in 96-well round-bottomed tissue culture plates. After incubation the plates were centrifuged and 100 $\mu$l of the resulting supernatant was transferred to flat-bottomed plates so that the absorbance (405 nm) of hemoglobin released from lysed cells could be quantified on a microplate spectrophotometer. Specific cytotoxic activities were calculated as percentages relative to maximum release ($4 \times 10^6$ RRBC/well in $H_2O$) and spontaneous release ($4 \times 10^6$ RRBC/well in TBS-Ca, no hemocytes) values.

### *Statistical analysis*

Nontreated controls were included in all experiments. A minimum of four tunicates were tested for each dose

**Table I**

*Percentage viabilities of hemocytes after 8 days of* in vitro *or aquarium exposure to a variety of toxicants*

| Treatment | % viable cells ± SEM ($n \geq 4$) | |
|---|---|---|
| | *In vitro* | Aquarium |
| Control (no treatment) | 87.3 ± 1.6 | 95.0 ± 0.6 |
| TBT (10 μg/l) | 89.3 ± 3.4 | 94.3 ± 1.2[a] |
| Cu (0.5 μg/ml) | 75.1 ± 2.1[β] | 93.1 ± 2.3 |
| Creosote (1%) | 72.6 ± 4.8[β] | 96.6 ± 0.7 |

[a] Tested after 6-day exposure.
[β] $P < 0.05$ *vs.* nontreated control.

and time point in aquarium trials. More than three replicates were tested for each dose and time point during *in vitro* exposures. The statistical significance of differences between treatments was determined by Student's two-tailed *t*-test using the Statview SE+ (Abacus Concepts Inc, Berkeley, CA) and Statworks (Cricket Software Inc., Philadelphia, PA) statistical packages.

## Results

### *Effects of toxicants on hemocyte viability*

The viability of hemocytes emigrating from *in vitro* pharyngeal cultures was decreased by exposure to copper or creosote (Table I). Decreases in viability were dose-dependent, with concentrations greater than 0.001% creosote or 0.05 μg/ml copper significantly lowering hemocyte survival after 8 days of exposure ($P < 0.05$ *vs.* nontreated controls; Fig. 1). Ten percent creosote or 50 μg/ml copper decreased viability by 37% and 48% relative to nontreated controls.

In contrast, TBT did not significantly alter hemocyte viability at any of the doses or times tested during *in vitro* exposures ($P > 0.05$). Moreover, the viabilities of hemocytes harvested from tunicates that had been exposed to creosote, copper, or TBT for up to 9 days in aquaria did not differ significantly from those of nontreated controls ($P > 0.05$; Table I).

### *Alteration of proliferative activity*

Exposure to toxicants profoundly affected the uptake of $^3$H-thymidine by pharyngeal explants (Table II). Exposure to TBT *in vitro* was the only treatment that did not significantly alter proliferation at any of the doses or times tested ($P > 0.05$; Fig. 2A). TBT treatment in aquaria reduced proliferative activity by as much as 62% after 6 days of exposure (10 μg/l; Fig. 2A) and copper inhibited $^3$H-thymidine incorporation by up to 82%

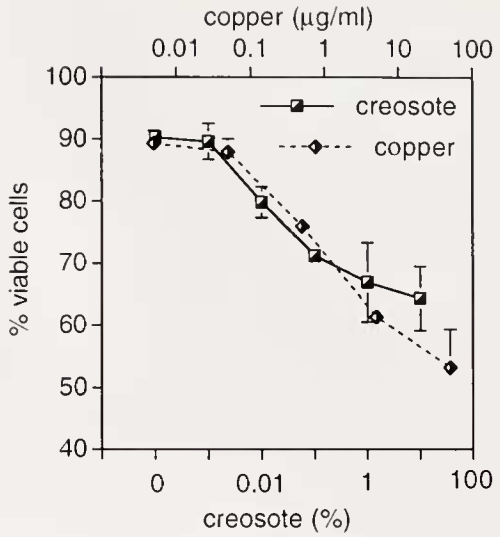


**Figure 1.** The percentage of viable cells (±SEM, $n \geq 4$) in the population of hemocytes that had emigrated from tunicate pharyngeal explants after 8 days of *in vitro* exposure to various doses of creosote and copper.

(0.5 μg/ml, day 8; Table II) when compared to nontreated controls.

The effects of creosote on proliferation were both time- and dose-dependent. After 4 days of exposure to high doses of creosote in aquaria (≥1%), proliferative activity was significantly increased relative to nontreated controls ($P < 0.05$; Fig. 2B). In contrast, treatment with 0.5% creosote had no effect after 4 days of exposure but significantly enhanced $^3$H-thymidine uptake (252% *vs.* controls) after 8 days. At this latter time point, proliferative activity in tunicates exposed to high doses (≥1% creosote) had subsided. Significant effects of TBT on proliferation were evident after 4 days of exposure and did not subside over time (Fig. 2A).

**Table II**

*Effect on hematopoietic cell proliferation of exposure for 8 days to various toxicants either* in vitro *or in aquaria; data are represented as percentages of $^3$H-thymidine uptake detected in nontreated controls*

| Treatment | % of nontreated control[γ] ± SEM ($n \geq 6$) | |
|---|---|---|
| | *In vitro* | Aquarium |
| TBT (10 μg/l) | 124.1 ± 23.2[α] | 38.8 ± 7.1[β] |
| Cu (0.5 μg/ml) | 31.6 ± 4.2 | 17.7 ± 4.8 |
| Creosote (0.5%) | 71.3 ± 8.0 | 252.1 ± 2.1 |

[α] $P > 0.05$ *vs.* nontreated controls.
[β] 6-day exposure.
[γ] Mean $^3$H-thymidine uptake by nontreated controls = 987 ± 23 cpm.

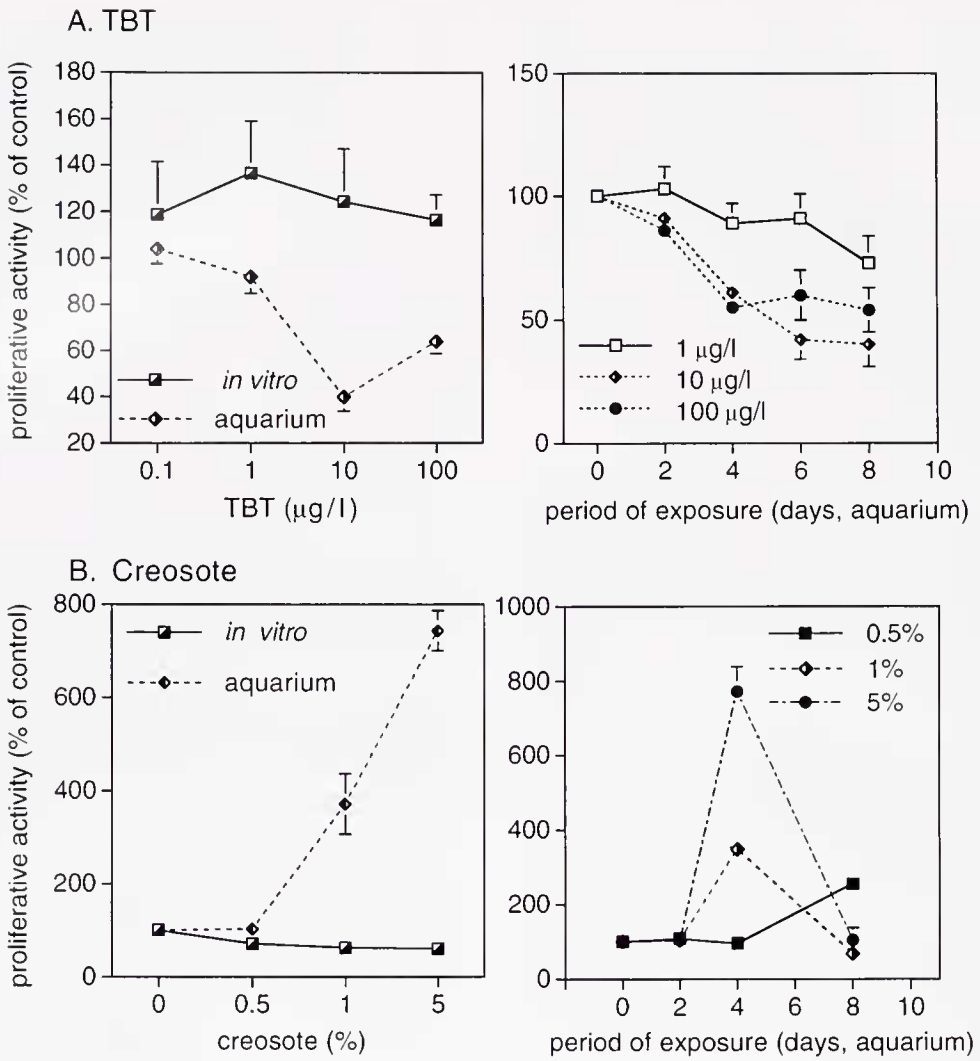


**Figure 2.** Proliferative activity (percentage of nontreated control values ± SEM, $n \geq 4$) in pharyngeal explants that were treated with various concentrations of (A) TBT or (B) creosote by either *in vitro* or aquarium exposure and harvested from tunicates after various periods of exposure. Uptake by controls: Day 2 = 322 ± 47 cpm; Day 4 = 874 ± 71 cpm; Day 8 = 944 ± 79 cpm.

*Effects of toxicants on phagocytosis*

Incubation of hemocytes with copper *in vitro* did not significantly ($P > 0.05$) alter phagocytic activity (Table III). However, copper substantially decreased phagocytosis (62% *vs.* control, 8 days, 0.5 µg/ml) during aquarium exposures. Exposure to 10 µg/l TBT *in vitro* significantly ($P < 0.05$) stimulated phagocytosis, whereas hemocytes taken from tunicates that had been treated with TBT in aquaria had lower phagocytic activities than those of nontreated controls (Table III). Creosote exposure significantly reduced phagocytosis after *in vitro* treatment, but greatly enhanced the phagocytic activity of hemocytes from tunicates exposed in aquaria (Table III).

The effects of *in vitro* TBT treatment on phagocytosis were dose-dependent (Fig. 3A). *In vitro* doses of TBT ranging from 1 to 10 µg/l enhanced phagocytosis by up to 220% relative to nontreated controls ($P < 0.05$), whereas a higher dose (100 µg/l) reduced phagocytic activity to 51% of its normal level ($P < 0.05$ *vs.* control). Other treatments consistently enhanced (creosote in aquaria) or inhibited (creosote *in vitro*, TBT in aquaria) phagocytosis over the range of doses tested (Fig. 3A).

Modulation of phagocytic activity by toxicants was also time-dependent (Fig. 3B). Alterations of phagocytosis resulting from treatment with both TBT and creosote in aquaria were most pronounced after short periods of exposure (creosote = 314% of control value, day 2; TBT

**Table III**

*Phagocytic activities, relative to nontreated controls, of tunicate hemocytes that had been exposed to various toxicants for either 24 h* in vitro *or 8 days in aquaria*

| | % of nontreated control[γ] ± SEM ($n \geq 4$) | |
|---|---|---|
| Treatment | *In vitro* | Aquarium |
| TBT (10 μg/l) | 220.1 ± 3.2 | 75.6 ± 3.1[α] |
| Cu (0.5 μg/ml) | 89.2 ± 8.4[β] | 37.4 ± 15.7 |
| Creosote (1%) | 56.8 ± 12.5 | 196.5 ± 21.1 |

[α] 6-day exposure.
[β] $P > 0.05$ *vs.* nontreated controls.
[γ] Mean phagocytic activity in nontreated controls = 0.43 ± 0.11 yeast ingested per hemocyte.

= 69.4% of control value, day 3) and returned to levels similar to control values after longer treatment times (creosote = 148% of control, 8 days; TBT = 105% of controls, 9 days).

*Cytotoxic activities after toxicant treatment*

Creosote was the only toxicant that significantly affected the cytotoxic activity of hemocytes toward either K562 or RRBC targets (Table IV). Exposure to creosote in aquaria inhibited cytotoxic activity toward K562 cells in a dose-dependent fashion (Fig. 4A). Treatment with 5% creosote for 8 days reduced anti-K562 cytotoxicity to levels that did not differ significantly from those of negative controls in which hemocytes and K562 cells were mixed at 4°C ($P > 0.05$; Fig. 4A). Significant ($P < 0.05$) inhibition of cytotoxic responses toward K562 cells was evident at all of the creosote doses tested (Fig. 4A). A different pattern emerged in assays that used RRBC as targets. Exposure to creosote doses greater than 0.5% for 8 days significantly enhanced the cytotoxic activity of hemocytes toward RRBC (Fig. 4A).

Figure 4B shows that the inhibitory effect of creosote on cytotoxicity toward K562 cells was reversible. The cytotoxic activity of hemocytes from tunicates that had been exposed to 5% creosote for 4 days and then transferred to toxicant-free seawater for a further 4 days (13.9% specific cytotoxicity) did not differ significantly from those of nontreated controls (15.3% specific cytotoxicity; $P > 0.05$). However, it was significantly greater than the cytotoxic activities evident among hemocytes taken from tunicates that had been exposed to 5% creosote continuously for 8 days (5.4% specific cytotoxicity; $P < 0.05$).

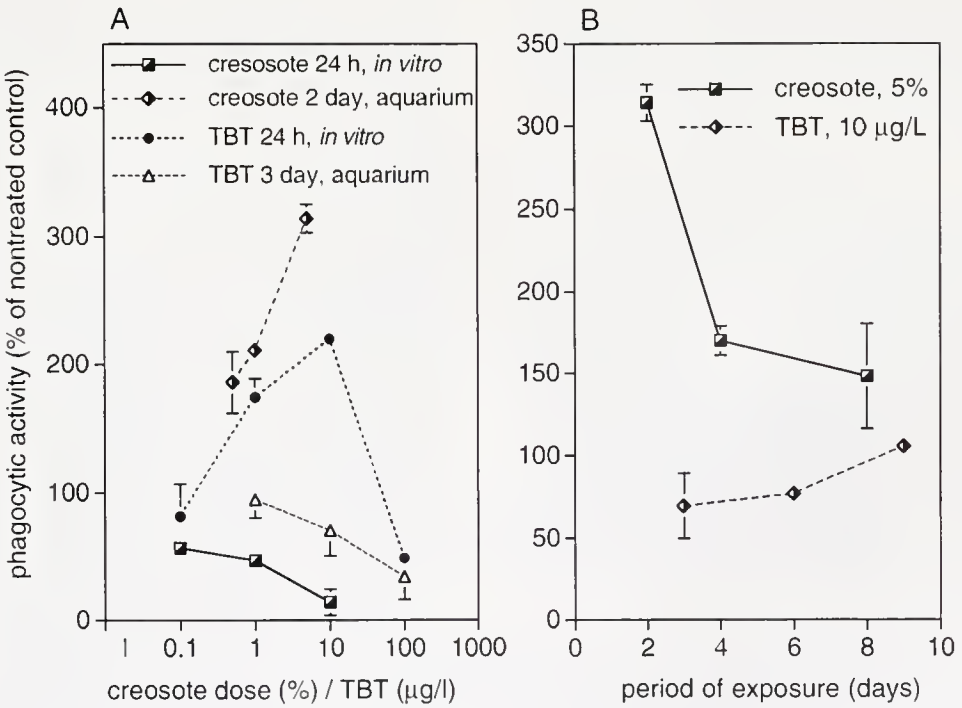


**Figure 3.** Phagocytic activities (percentage of nontreated control values ± SEM, $n \geq 4$) of tunicate hemocytes that were (A) exposed to various doses of creosote or TBT overnight *in vitro* or harvested from tunicates that had been treated with creosote or TBT for 2 or 3 days in aquaria, or (B) harvested from tunicates after various periods of exposure to 5% creosote or 10 μg/l TBT in aquaria.

**Table IV**

*Effect of treatment with various toxicants either overnight (*in vitro *exposure) or for 8 days (aquarium exposures) on cytotoxic activity of tunicate hemocytes toward K-562 or rabbit red blood cells (RRBC)*

| | % of nontreated control[γ] ± SEM ($n \geq 4$) | | |
|---|---|---|---|
| Treatment | *In vitro*/ K562 | Aquarium/ RRBC | Aquarium/ K562 |
| TBT (10 μg/l) | nt[α] | 121.1 ± 14.3 | 119.0 ± 26.9[β] |
| Cu (0.5 μg/ml) | 85.8 ± 5.2 | 96.8 ± 6.4 | 108.1 ± 21.3 |
| Creosote (1%) | nt | 138.9 ± 6.9[δ] | 53.6 ± 15.4[δ] |

[α] Not tested.
[β] 6-day exposure.
[δ] $P < 0.05$ *vs.* nontreated control.
[γ] Mean cytotoxic activities for nontreated controls = 43.6 ± 6.1 for K562 and 21.2 ± 3.1 for RRBC.

### *Effect of creosote on the frequency of hemocyte subpopulations*

Creosote was the only toxicant that affected the frequencies of hemocyte subpopulations in tunicates exposed to toxicants in aquaria (Fig. 5). Three distinct subpopulations of hemocyte were identified by analysis of forward angle *vs.* 90° light-scatter plots. They were designated small (low forward scatter), large (high forward scatter, low 90° scatter) and large granular (high forward and high 90° scatter) hemocytes. Treatment with creosote significantly decreased the frequency of large granular hemocytes relative to the other hemocyte subpopulations. The frequency of large granular hemocytes was reduced by two-thirds relative to nontreated controls after 4 days of exposure ($P < 0.05$; Fig 5A). This effect was apparent within 2 days of exposure to high doses of creosote (5%) and was evident for all of the doses tested within 4 days (Fig 5A). There was a corresponding increase in the frequency of small hemocytes: after 4 days about two times more small hemocytes were present within treated tunicates than in nontreated controls (Fig 5B). The frequency of large hemocytes did not vary from control levels at any of the doses or times tested (Fig 5B).

## Discussion

This study has demonstrated that sublethal contamination by common estuarine pollutants can significantly alter innate immunological reactions in tunicates. The three compounds tested here (TBT, copper, and creosote) are particularly relevant. All three are major components of antifouling films that are used to prevent the growth of sessile invertebrates. TBT can leach from marine paints and accumulate to hazardous levels in harbors and marinas (Bryan *et al.*, 1986; Huggett *et al.*, 1986). It is known to inhibit chemiluminescence responses, chemotactic activity, phagocytic oxidative bursts and phagocytosis by fish leukocytes (Rice and Weeks, 1991; Warinner *et al.*, 1988; Weeks *et al.*, 1986).

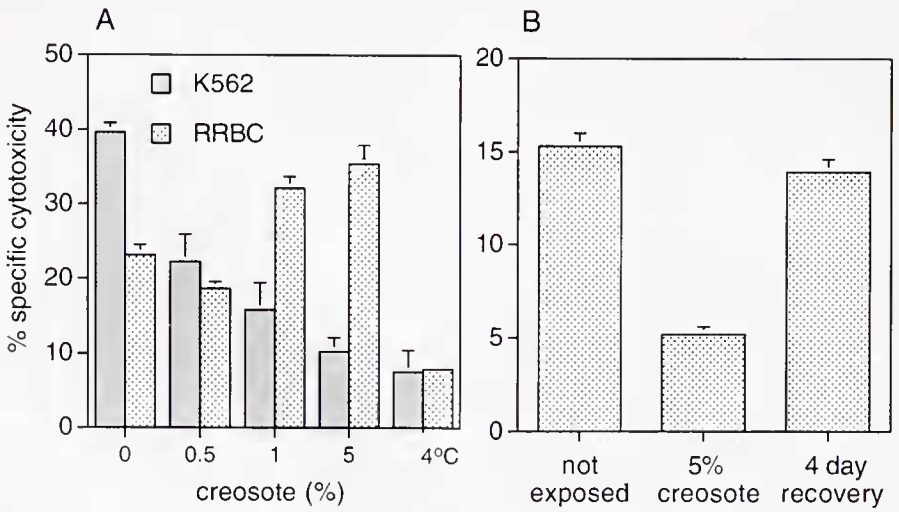


**Figure 4.** (A) Cytotoxic activities (percentage of nontreated control values ± SEM, $n = 4$) of hemocytes taken from tunicates that were exposed to various doses of creosote in aquaria for 8 days; 4°C represents hemocytes from nontreated controls that were mixed with targets at 4°C to suppress cytotoxic activity. (B) Cytotoxic activities (percent specific cytotoxicity toward K562 cells ± SEM, $n = 4$) of hemocytes harvested from tunicates that had either been held in aquaria without creosote (not exposed), exposed to 5% creosote in aquaria for 8 days (5% creosote), or exposed to 5% creosote in aquaria for 4 days and then transferred to fresh seawater without creosote for a further 4 days (4-day recovery).

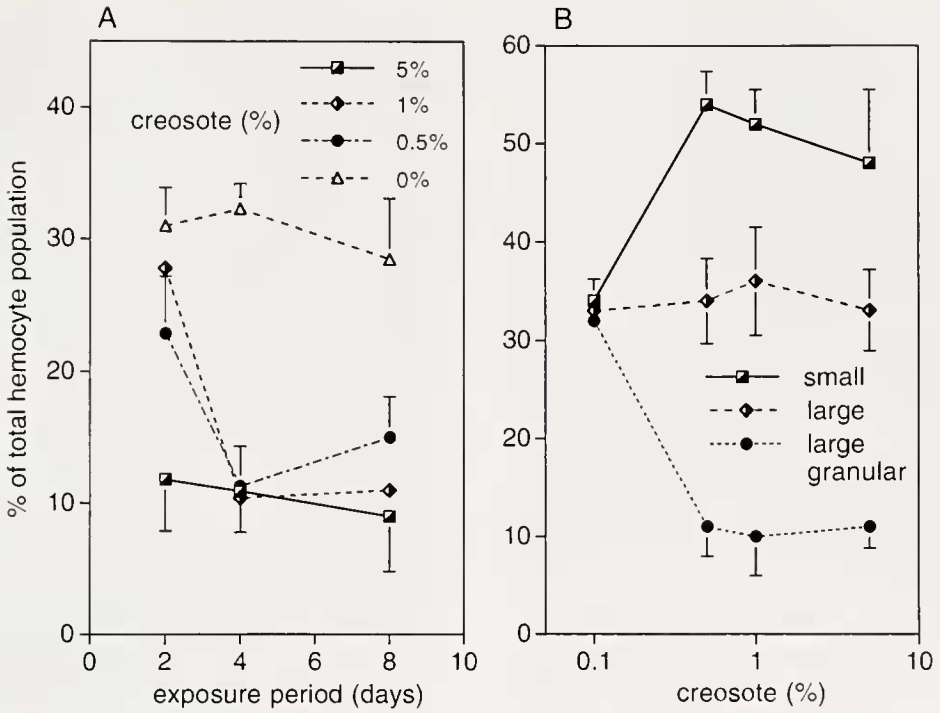


**Figure 5.** Frequencies (percentages of the total hemocyte population ± SEM, $n = 4$) of (A) large granular hemocytes in hemolymph harvested from tunicates after various periods of exposure to a range of creosote concentrations in aquaria, or (B) small, large, and large granular hemocytes harvested from tunicates that been exposed to various doses of creosote for 4 days in aquaria.

Although the use of TBT on pleasure craft is now prohibited widely, it is still applied to larger vessels and remains a common harbor contaminant (NSW Environment Protection Authority, pers. comm.; Stebbing, 1985). For use on small craft, TBT has been superseded by copper-based antifouling products. Like TBT, copper is known to have substantial immunological effects on aquatic organisms at subacute doses (Roales and Perlmutter, 1977). Creosote is a hydrocarbon-based protective coating that is frequently used on pylons, wharves, and netting. It is composed of about 85% PAH, 10% phenolic compounds, and 5% heterocyclic compounds (Faisal *et al.*, 1991). PAHs in particular have been associated with a variety of physiological effects including carcinogenesis and the alteration of immune reactivity in humans and other animals (Faisal *et al.*, 1991; National Research Council, USA, 1972). In fish, PAHs are responsible for the development of eye lens cataracts, gill necrosis, degeneration of renal epithelia, and neoplasia. They can also inhibit macrophage function, cellular cytotoxicity, and cellular proliferation (Faisal *et al.*, 1991). The range of doses that were tested in this study include levels that can frequently be found in the environment. TBT concentrations >2 μg/l have been detected in marine areas (Waldcock and Miller, 1983). PAH levels greater than 10 times the maximum dose used here are reportedly common for harbor waters, and levels of copper up to 2.5 μg/ml have been detected in heavily utilized environments (Hyland and Scheider, 1976; Pluarg International Joint Commission, 1978; Waldhauer *et al.*, 1978).

Only high doses of copper (≥5 μg/ml) were acutely lethal to tunicates. However, all of the toxicants tested here exerted powerful effects on at least some immunological reactions (summarized in Table V). The ability of toxicants, particularly creosote, to simultaneously affect a variety of apparently unrelated parameters, such as phagocytosis, proliferation, and cellular cytotoxicity, does not simply reflect general morbidity or metabolic downturn resulting from toxicant poisoning. The observation that toxicants enhanced some hemocyte-mediated responses but inhibited or had no effect on others confirms that tunicate cells were not generally inactivated by toxicant treatment. There is also no evidence that any of the toxicants affected the viability of cells from tunicates that were exposed in aquaria (except after death at lethal doses of copper), even though both creosote and copper decreased hemocyte viability *in vitro*. This discrepancy has three plausible explanations. First, the observed differences in hemocyte viabilities may

have been due to the rapid clearance of dead hemocytes *in vivo*. Those dead cells may not have appeared in the circulating hemolymph, and so may not have been detected in viability assays. Second, tunicates could possess mechanisms to detoxify, sequester, or prevent the penetration of copper and creosote *in vivo*. This possibility is not, however, supported by the observation that some immunological reactions were similarly affected by identical doses of toxicants applied *in vitro* and in aquaria (*e.g.*, copper's inhibitory effect on cell proliferation). Third, differences between *in vitro* and aquarium trials might have been due to the existence of compensatory or interactive mechanisms that cannot operate in isolated *in vitro* systems.

The latter explanation is supported by differences that were evident between the effects of *in vitro* and aquarium exposures on immunological parameters such as phagocytosis, cell proliferation, and cytotoxicity. For instance, *in vitro* creosote treatment inhibited phagocytic activity and cell proliferation, whereas tunicates treated with creosote in aquaria had an enhanced capacity for phagocytosis and a transient increase in proliferative activity. Such contrasting results indicate that some effects *in vivo* may result from interactive mechanisms rather than from direct toxicity toward the response being examined. Creosote poisoning, for instance, may have stimulated regulatory activity that specifically enhanced phagocytosis and proliferation. Mechanisms that are capable of such cellular regulation are well characterized in tunicates. Regulatory molecules in the hemolymph can enhance phagocytosis and cell proliferation in a manner analogous to the activities of vertebrate cytokines (Beck *et al.*, 1993; Raftos, 1994; Raftos *et al.*, 1991).

The data also suggest that tunicates have mechanisms that can confer some degree of adaptive protection, and allow recuperation, from the effects of toxicants. The existence of mechanisms for adaptive protection is indicated by the kinetics of toxicant poisoning. Alterations in the levels of phagocytosis among tunicates treated with creosote or TBT in aquaria were short lived. Phagocytic activity returned to more-or-less normal levels after 8 days of continuous exposure. This amelioration can be explained in two ways. First, creosote and TBT may have become detoxified over the 8-day exposure period. Second, adaptive mechanisms that reduced the effect of toxicants on phagocytosis may have been activated. The latter possibility is supported by evidence indicating that both creosote and TBT retain their toxicity toward other immunological reactions for at least 8 days. The existence of processes that allow recuperation from the effects of toxicants is indicated by the rapid recovery of cytotoxic activity when tunicates are transferred from contaminated to fresh seawater. There are many possible mechanisms by which toxicants could gain their effects, or by which tunicates might adaptively ameliorate those effects. For instance, the observed recuperation of some reactions from poisoning may reflect the existence of inducible detoxication mechanisms that directly affect toxicant bioavailability. Inducible low molecular weight metal-binding proteins (metallothioneins) that can bind and detoxify a variety of metals such as copper and tin have been identified in many species (George, 1990).

An alternative explanation for the varied effects of toxicants is that alterations in the frequencies of specific effector cells are responsible both for altering immune functions and for the later recovery of those immune reactions from poisoning. In fish, it has been reported that the frequencies of specialized, ion-transporting chloride cells vary significantly during copper poisoning as those cells migrate to and from fixed tissues (Pelgrom *et al.*, 1995). Similarly, the frequency of large granular hemocytes in *S. plicata* decreased rapidly upon exposure to creosote. The large granular hemocyte population includes vesiculated cell types that have been implicated in wound repair and that become localized in fixed tissues during inflammation-like responses (Goodbody, 1974; Wright, 1981). These cells may have been sequestered in fixed tissues damaged during creosote treatment, and so may have altered the relative mix of hemocyte types in the hemolymph. The loss of large granular hemocytes from the circulation may also have activated compensatory hematopoietic cell proliferation. Such hematopoietic proliferation was detected directly by increased $^{3}$H-thymidine uptake and is also indicated by the specific increase observed in the frequency of small hemocytes, a population that includes immature hemoblasts derived from hematopoiesis (Ermak, 1982; Rinkevich and Rab-

**Table V**

*Summary of the effects of different toxicants on a variety of responses that were tested either* in vitro (vit) *or by aquarium exposure* (aqu)

| | Toxicant/treatment | | | | | |
|---|---|---|---|---|---|---|
| | TBT | | Creosote | | Copper | |
| Responses tested | vit | aqu | vit | aqu | vit | aqu |
| Cell viability | 0 | 0 | ↓ | 0 | ↓ | 0 |
| Phagocytosis | ↔ | ↓ | ↓ | ↑ | 0 | ↓ |
| Proliferation | 0 | ↓ | ↓ | ↔ | ↓ | ↓ |
| Cytotoxicity (K562) | — | 0 | — | ↓ | 0 | 0 |
| Cytotoxicity (RRBC) | — | 0 | — | ↑ | — | 0 |

↑ = enhanced by toxicant treatment; ↓ = inhibited by toxicant treatment; ↔ = enhanced or suppressed depending upon dose or time of exposure; 0 = no significant alteration relative to nontreated controls; — = not tested.

inowitz, 1993; Wright, 1981). The regulatory activity that may have activated cell proliferation might also have affected phagocytic activity. Tunicate cytokine-like molecules have pleiotropic effects that include the simultaneous activation of phagocytosis and proliferation (Beck *et al.*, 1993; Raftos, 1994; Raftos *et al.*, 1991). This role of altered hemocyte frequencies on immune functions remains speculative, but is currently being tested.

In conclusion, it is clear that environmental contaminants have profound effects on immunological reactions in tunicates. Those effects are unlikely to be the result of general morbidity, and they are not reflected by acute lethality. However, because of the implicit relationship between innate immune reactions and antipathogenic defenses, it is likely that the effects demonstrated here alter the capacity of tunicates to defend themselves against infection. The relevance of these effects to the viability of tunicate populations remains unclear. Little is known about the level of surveillance that is required by tunicates for survival. We are investigating that relationship between innate or natural immunological competance and long-term population health by testing the effects of environmental toxicants on the ability of tunicates to deal with artificial and natural infections.

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